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Effect of β-Lapachone on Superoxide Anion and Hydrogen Peroxide Production in *Trypanosoma cruzi*

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Addition of β-lapachone, an *o*-naphthoquinone endowed with trypanocidal properties to respiring *Trypanosoma cruzi* epimastigotes induced the release of O_2^- and H_2O_2 from the whole cells to the suspending medium. The same β-lapachone concentration (4 μM) that released H_2O_2 at maximal rate completely inhibited *T. cruzi* growth in a liquid medium. The position isomer, α-lapachone, did not stimulate O_2^- and H_2O_2 release, and did not inhibit epimastigote growth. β-Lapachone was able to stimulate H_2O_2 production by the epimastigote homogenate in the presence of NADH as reductant. The same effect was observed with the mitochondrial fraction supplemented with NADH, where β-lapachone enhanced the generation of O_2^- and H_2O_2 4.5- and 2.5-fold respectively. β-Lapachone also increased O_2^- and H_2O_2 production (2.5 and 2-fold respectively) by the microsomal fraction with NADPH as reductant. Cyanide-insensitive NADH and NADPH oxidation by the mitochondrial and microsomal fractions (quinone reductase activity) was stimulated to about the same extent by β-lapachone. α-Lapachone was unable to increase O_2^- and H_2O_2 production and quinone reductase activity of the mitochondrial and microsomal fractions.

β-Lapachone (3,4-dihydro-2,2-dimethyl-2*H*-naphtho[1,2-*b*]pyran-5,6-dione), a lipophilic cytotoxic *o*-naphthoquinone (Lima *et al.*, 1962; Santana *et al.*, 1968), is also an effective trypanocide, causing severe ultrastructural and metabolic alterations in *Trypanosoma cruzi*, the agent of Chagas' disease (Docampo *et al.*, 1977; Cruz *et al.*, 1978). β-Lapachone stimulates H_2O_2 generation and lipoperoxide formation in the culture (epimastigote) form of *T. cruzi* and the quinone-treated epimastigotes show a clear e.p.r. signal of the semiquinone, thus indicating the biological reduction of β-lapachone (Docampo *et al.*, 1978*a*). Since in biological systems naphthoquinone generates O_2^- and H_2O_2 (Misra & Fridovich, 1972*b*), and H_2O_2 is toxic for trypanosomatidae (Fulton & Spooner, 1956), we have compared the effect of β-lapachone and its biologically inactive position isomer α-lapachone (3,4-dihydro-2,2-dimethyl-2*H*-naphtho[2,3-*b*]pyran-5,10-dione) on O_2^- and H_2O_2 generation and growth of *T. cruzi* (Tulahuen strain) *in vitro*. In the present paper, we report a correlation between the ability of β- and α-lapachone to produce O_2^- and H_2O_2 and their action on the growth of *T. cruzi*.

Despite the fact that Chagas' disease, caused by *T. cruzi*, is an important health problem in Latin America, a therapeutic agent effective against all forms of the parasite has not yet been found. A rational approach to the design of a trypanocidal drug is the investigation of substances such as β-lapachone, which, by stimulating the production of toxic metabolites, may lead to a lethal situation for the parasite.

Materials and Methods

The Tulahuen strain of *T. cruzi* was grown in a liquid medium consisting of brain–heart infusion (Difco Laboratories, Detroit, MI, U.S.A.) 37g, haemin chlorhydrate (dissolved in triethanolamine) 20mg, and bovine serum, 100ml/litre (Warren, 1960). Epimastigote growth was determined by cell counting in a Neubauer chamber. Quinones in ethanolic solution were aseptically added to the culture medium. The corresponding controls showed no effect of the amount of added ethanol on epimastigote growth. For the preparation of subcellular fractions, the epimastigotes were disrupted by freezing at −16°C and thawing three times. The cells were suspended in 0.23M-mannitol/0.07M-sucrose/1mM-EDTA/10mM-Tris/HCl, pH 7.2, at 8mg of protein/ml, and were homogenized by several passages through a no. 24-gauge hypodermic needle attached to a syringe. The homogenates were fractionated in a Sorvall RC-2B centrifuge at 2°C. The fractions obtained were: (*a*) the nuclear–flagellar fraction, which amounted to 20% of the total protein; (*b*) the mitochondrial fraction, sedimented at 12000*g* for 10min,

which accounted for 40% of the total protein; (*c*) the microsomal fraction, sedimented at 105000*g* for 45min, which accounted for 7% of the protein; (*d*) the supernatant, containing 33% of total protein. The specific activities of succinate dehydrogenase, selected as marker enzyme and determined by the Arrigoni & Singer (1962) assay, were 4.3, 13.6, 5.1 and 0nmol of succinate/min per mg of protein in fractions (*a*), (*b*), (*c*) and (*d*) respectively. The protein content of cell suspensions and subcellular fractions was determined by the biuret assay (Gornall *et al.*, 1949) in the presence of 0.2% sodium deoxycholate.

The rates of H_2O_2 generation were determined by measuring the rate of horseradish peroxidase H_2O_2 formation by dual-wavelength spectrophotometry at 417–402nm ($\Delta\varepsilon = 50$ litre·mmol^{-1}·cm^{-1}; Boveris *et al.*, 1972). The rates of O_2^- generation were determined by measuring adrenochrome formation by dual-wavelength spectrophotometry at 485–575nm ($\Delta\varepsilon = 2.97$ litre·mmol^{-1}·cm^{-1}; Misra & Fridovich, 1972*a*; Cadenas *et al.*, 1977). The rates of O_2^- generation were calculated from the superoxide dismutase-sensitive rate of adrenochrome production, which was usually higher than 95% of the total rate of adrenochrome formation. Quinone reductase activity was measured by following either NADH or NADPH oxidation at 340nm in a Gilford 2000 spectrophotometer. Mitochondrial and microsomal fractions (0.2–0.5mg of protein/ml) were suspended in 50mM-potassium phosphate buffer, pH7.4, containing 1mM-KCN, 100μM-NADH (or NADP) and quinone (0–40 μM). All enzyme reactions were measured in a thermostatically controlled cell compartment at 30°C. The rates of generation of O_2^- and H_2O_2 by quinols in buffered solutions were measured as described above. Quinol concentrations were determined by recording their u.v. absorption spectra (Cadenas *et al.*, 1977) with a Beckman DK-2 spectrophotometer. The second-order reaction constants for the production of O_2^- (k_3) and H_2O_2 (k_4) in the autoxidation reaction of quinols were calculated from the linear relationship between the amount of quinol and the rate of O_2^- and H_2O_2 generation. Quinols were diluted to 1–30μM in 50mM-Tris/Mops(4-morpholinepropanesulphonic acid), pH7.4, as indicated previously (Cadenas *et al.*, 1977).

Chemicals

β- and α-lapachone were provided by Dr. S. Albónico, Department of Pharmaceutical Sciences, School of Pharmacy and Biochemistry, University of Buenos Aires. Quinone concentration and purity were determined by recording their u.v. absorption spectra in a Beckman DK-2 spectrophotometer. β-Lapachone has an ε_{258} of 40 litre·mmol^{-1}·cm^{-1} and an $\varepsilon_{440} = 3.1$ litre·mmol^{-1}·cm^{-1} and α-lapachone has an $\varepsilon_{250} = 31$ litre·mmol·cm^{-1}, both in ethanolic solution (Fig. 1). Quinols were prepared by addition of 0.1ml of a KBH_4 solution (30mg of KBH_4/ml of 0.1M-KOH) to 0.8ml of an approx. 10mM-quinone solution in dimethylformamide/water (3:7, v/v). HCl (0.1ml of 0.2M) was added to decompose excess borohydride, and the final clear solution was supplemented with 10μl of 0.1M-EDTA.

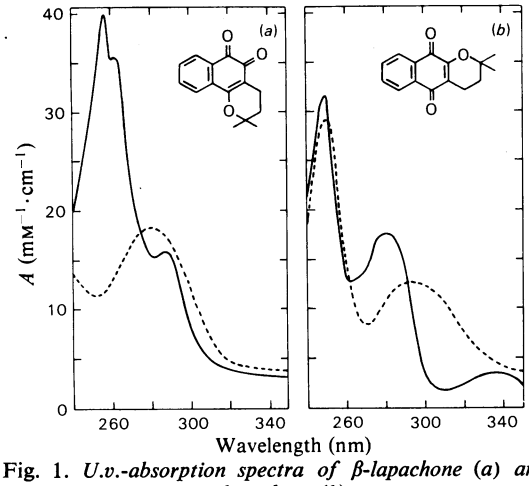

Fig. 1. *U.v.-absorption spectra of β-lapachone (a) and α-lapachone (b)*

——, Oxidized form; ----, reduced form. A 1cm light-path was used. Other conditions were as described in the Materials and Methods section.

NADH, NADPH, adrenaline bitartrate, horseradish peroxidase type VI and bovine superoxide dismutase were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A.

Results

Observations with intact epimastigotes and homogenates

Respiring *T. cruzi* epimastigotes did not release either O_2^- or H_2O_2 to the suspending medium as detected by the adrenochrome assay for O_2^- and by H_2O_2–horseradish peroxidase formation for H_2O_2 (Fig. 2*a* and 2*b*). The lack of release of H_2O_2 from whole cells had been previously observed in spite of the active H_2O_2 generation of *T. cruzi* cells (Boveris & Stoppani, 1977), which implies the operation of an intracellular H_2O_2-detoxifying system (Docampo *et al.*, 1976). Addition of β-lapachone initiated an immediate production of adrenochrome, which was sensitive to superoxide dismutase, indicating O_2^- generation (Fig. 2*b*). At low β-lapachone concentrations (up to 4μM) O_2^- and H_2O_2 were released in the ratio of 1 O_2^- to 3 H_2O_2 (Fig. 3) and at the higher quinone concentrations, O_2^- release kept a linear relationship with quinone concentration. In contrast

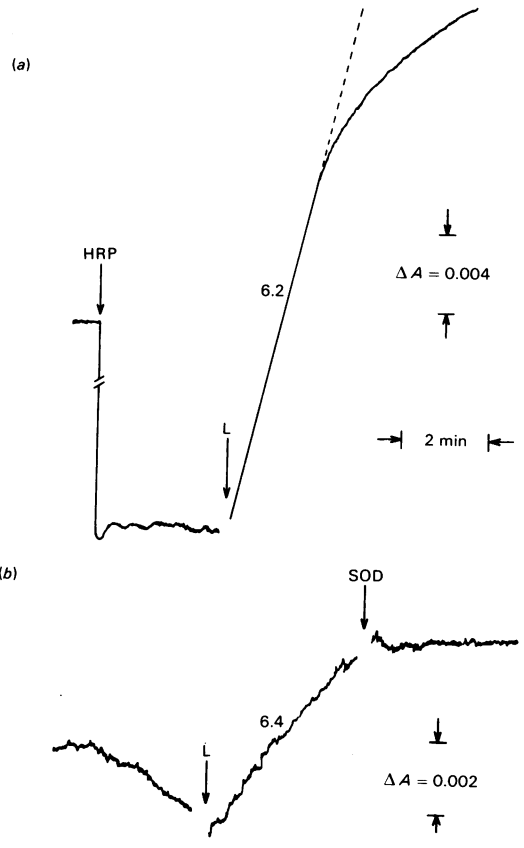

Fig. 2. *Production of H_2O_2 and O_2^- in T. cruzi epimastigotes on addition of β-lapachone*

(*a*) Production of H_2O_2 measured at 417–402nm. The incubation mixture contained: 154mM-NaCl, 20mM-potassium phosphate buffer, pH7.2, 0.5μM-horseradish peroxidase (HRP), 0.5mg of cell protein/ml. The value against the trace indicates H_2O_2 released/min per 10^6 cells (in pmol). (*b*) Production of O_2^- measured at 485–575nm. The incubation mixture contained: 154mM-NaCl, 20mM-potassium phosphate buffer, pH7.2, 1mM-adrenaline, 1.75mg of cell protein/ml. L, 13μM-β-lapachone. The value against the trace indicates pmol of O_2^- released/min per 10^6 cells. Abbreviations used: L, 5μM-β-lapachone; SOD, 5μg of superoxide dismutase/ml.

with these results, H_2O_2 release reached a plateau and even slightly decreased. The plateau formation could be real and reflect saturation of intracellular superoxide dismutase (Boveris & Stoppani, 1977), a possibility that is compatible with the relatively short diffusional distances between the mitochondrial arms (where much of O_2^- generation occurs) and the surface of the cell (Paulin, 1975; Meyer & de Souza, 1976; Docampo *et al.*, 1977). However, it could also reflect an interference of β-lapachone-reduced forms with the rate of formation of the horseradish peroxidase–H_2O_2 complex (the spectrophotometric indicator), since quinols can act as hydrogen donors for the peroxidase reaction (Yamazaki *et al.*, 1960). At variance to these results, α-lapachone was unable to produce any significant release of O_2^- and H_2O_2 from the epimastigotes (Fig. 3).

T. cruzi homogenates supplemented with reduced nicotinamide nucleotides are effective sources of H_2O_2 (Boveris & Stoppani, 1977). Addition of β-lapachone increased H_2O_2 production by these homogenates significantly (about 3-fold the control rate at 30μM-β-lapachone; Fig. 3, inset). The reason for the declining H_2O_2 rates at the higher quinone concentration is probably the abovementioned quinol interference with the horseradish peroxidase–H_2O_2 complex. A similar but lesser stimulation of H_2O_2 generation by β-lapachone was observed in the presence of NADPH as reductant (Fig. 3, inset). In contrast with the effects of β-lapachone, α-lapachone did not increase H_2O_2 production by the homogenate, in good agreement with its lack of effect in stimulating O_2^- or H_2O_2 release from epimastigotes (Fig. 3 and Fig. 3, inset). The stimulation by β-lapachone of H_2O_2 production was about twice that with the homogenate as with the intact epimastigotes. Thus with intact epimastigotes 5μM-β-lapachone induced the release of 0.5nmol of H_2O_2/min per mg of protein (Fig. 3), whereas with the homogenate the same quinone concentration increased H_2O_2 production by 1nmol/min per mg of protein (Fig. 3, inset). This difference is consistent with the existence of an intracellular H_2O_2-utilizing system (Docampo *et al.*, 1976). In this connection, it is worth recalling that addition of β-lapachone also increased superoxide production by *T. cruzi* amastigote and epimastigote homogenates (Sonya strain) (Docampo *et al.*, 1978*c*).

Observations with subcellular fractions

Fractionation of *T. cruzi* homogenates by differential centrifugation yields fractions that contain fragments derived from the intracellular organelles. Although these fractions are far from possessing the purity of those obtained from mammalian tissues, they are fairly well characterized. Mitochondrial fractions from *T. cruzi* show NADH oxidase, NADH dehydrogenase and succinate dehydrogenase activities, and contain a cytochrome system with cytochromes $a + a_3$, b and c_{558} (Boiso & Stoppani, 1971; Agosin *et al.*, 1976; Docampo *et al.*, 1978*b*). Microsomal fractions show an antimycin-insensitive NADH–cytochrome *c* reductase activity and contain cytochrome *P*-450 (Boiso & Stoppani, 1971; Agosin *et al.*, 1976).

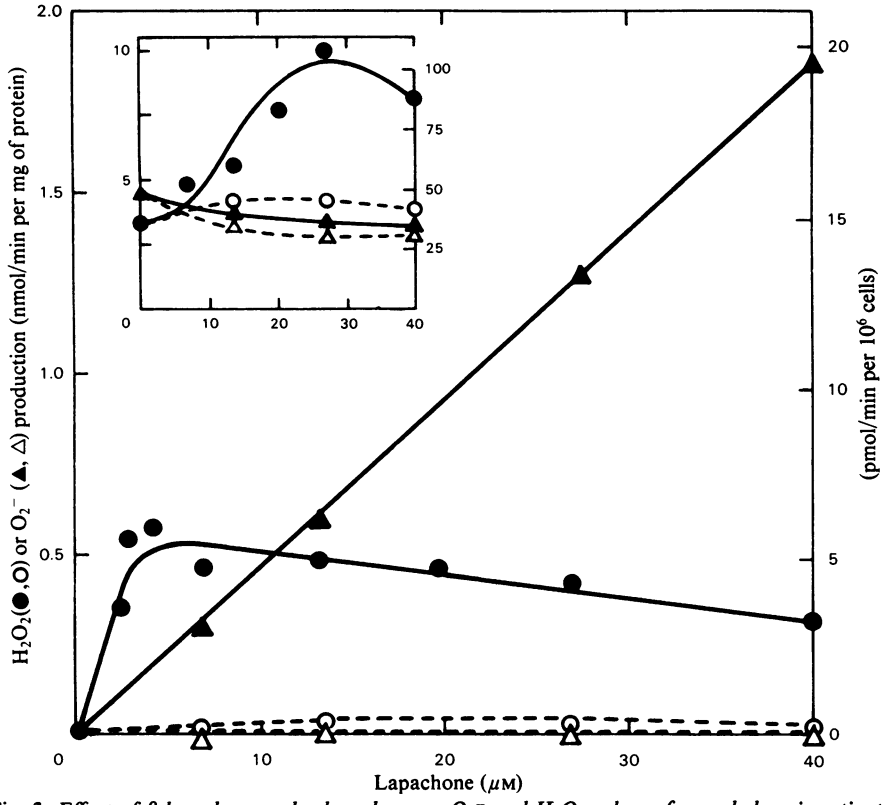

Fig. 3. *Effect of β-lapachone and α-lapachone on O_2^- and H_2O_2 release from whole epimastigotes*
Experimental conditions were as in Fig. 2. ▲, O_2^- release with β-lapachone; ●, H_2O_2 release with β-lapachone; △, O_2^- release with α-lapachone; ○, H_2O_2 release with α-lapachone. The inset shows the effect of β-lapachone and α-lapachone on H_2O_2 production by the homogenate of *T. cruzi* epimastigotes. The incubation mixture contained: 130mM-KCl, 20mM-potassium phosphate buffer, pH7.2, 40mM-NADH or -NADPH, 0.6μM-horseradish peroxidase, 0.1–0.2mg of homogenate protein/ml. ●, NADH and β-lapachone; ▲, NADPH and β-lapachone; ○, NADH and α-lapachone; △, NADPH and α-lapachone.

In good agreement with previous observations (Boveris & Stoppani, 1977), the mitochondrial fraction of *T. cruzi* actively generated H_2O_2 in the presence of NADH (Fig. 4*a*). Use of reduced nicotinamide nucleotides as electron donors was compulsory since, at variance with other mitochondrial preparations (Boveris & Chance, 1973; Boveris & Cadenas, 1975; Boveris *et al.*, 1976; Boveris, 1977), with *T. cruzi* mitochondrial fragments, succinate (and antimycin) do not modify the rate of H_2O_2 production. Addition of β-lapachone to the mitochondrial fraction supplemented with NADH, stimulated O_2^- production linearly up to 40μM-quinone, at which concentration the basal rate was increased 4.5 times (Fig. 4*a*). Production of H_2O_2 was stimulated about 2.5 times with a maximum at about 30μM-quinone (Fig. 4*a*). Lesser effects were observed with NADPH as reductant. Addition of α-lapachone did not stimulate either O_2^- or H_2O_2 production in the presence of either NADH or NADPH as reductant (results not shown). The effect of β-lapachone on H_2O_2 production with the mitochondrial fraction was in accordance with that observed with the homogenate, for NADH was more effective than NADPH as substrate and saturation was observed at about 30μM-quinone.

Production of O_2^- and H_2O_2 by the microsomal fraction was also stimulated by β-lapachone (Fig. 4*b*). The results correspond to our best microsomal preparation, judging by the nicotinamide nucleotide specificity for O_2^- and H_2O_2 production. In the presence of NADPH, the quinone was able to stimulate O_2^- and H_2O_2 generation by about 2-fold, the maximal stimulation being reached at about 20μM-β-lapachone. Much lesser effects were observed in the presence of NADH. Addition of α-lapachone to

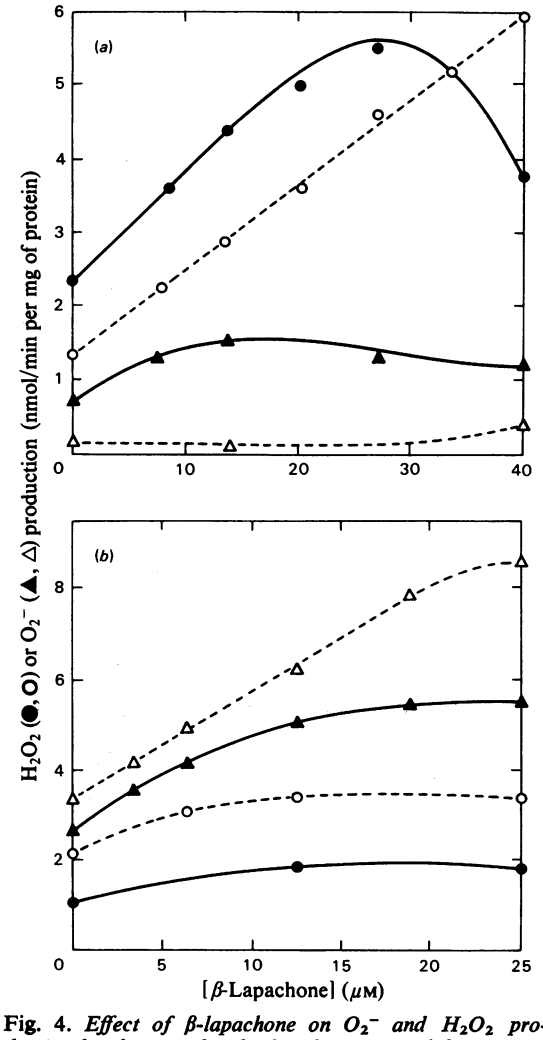

Fig. 4. *Effect of β-lapachone on O_2^- and H_2O_2 production by the mitochondrial and microsomal fractions of T. cruzi*

(*a*) Experiment with the mitochondrial fraction. The incubation mixture contained: 130mM-KCl, 20mM-potassium phosphate buffer, pH7.2, either 0.6μM-horseradish peroxidase or 1mM-adrenaline for H_2O_2 or O_2^- measurements respectively, 40μM-NADH or -NADPH, 0.1–0.4mg of protein/ml. ●, H_2O_2 production with NADH; ○, O_2^- production with NADH: ▲, H_2O_2 production with NADPH, △, O_2^- production with NADPH. (*b*) Experiment with the microsomal fraction; the conditions and symbols as for (*a*).

the microsomal preparations did not produce any effect on O_2^- and H_2O_2 production with either NADPH or NADH as substrate (results not shown).

The lapachone reductase activities of the mitochondrial and microsomal fraction were determined with NADH or NADPH as electron donors in the presence of cyanide and quinone (Fig. 5). The rate of NADH-oxidation by mitochondrial fragments was stimulated about 3-fold by 40μM-β-lapachone, whereas NADPH oxidation was less stimulated. It must be noted that α-lapachone did not stimulate NADH or NADPH oxidation by the mitochondrial fragments. Similar effects of β- and α-lapachone were found when the quinone reductase activity of microsomal preparations was determined. In this case, NADPH was more effective than NADH as reductant (Fig. 5, inset). β-Lapachone at 20–25μM was able to increase the cyanide-insensitive NADPH oxidation by the microsomal fraction about 5-fold, whereas NADH oxidation was less stimulated. α-Lapachone had no effect on the cyanide-insensitive NADH or NADPH oxidation by microsomal fragments.

The rates of nicotinamide nucleotide oxidation by the mitochondrial and microsomal fractions (Fig. 5) were 90–180% higher than the rates of H_2O_2 production by the same fractions, irrespective of β-lapachone addition (Figs. 4*a* and 4*b*). The relatively lower rates of H_2O_2 production are understandable considering that (*a*) some reduced nicotinamide nucleotide oxidation did not lead to H_2O_2 formation and (*b*) the horseradish peroxidase–H_2O_2 assay slightly underestimated H_2O_2 production (Boveris *et al.*, 1977).

The increased rate of O_2^- and H_2O_2 production after β-lapachone addition to the *T. cruzi* preparation involved the autoxidation of the corresponding quinols. Consequently the rate of the autoxidation reactions was measured and is expressed in Table 1 as the second-order reaction constants corresponding to eqns. (1) and (2)

$$d[O_2^-]/dt = k_3[LH_2][O_2] \quad (1)$$

$$d[H_2O_2]/dt = k_4[LH_2][O_2] \quad (2)$$

(menadiol is included for purposes of comparison). In spite of its more positive E_0', reduced β-lapachone was much more effective than α-lapachone as H_2O_2 generator, a characteristic derived from its *o*-quinone nature. For the other two *p*-quinones, namely, α-lapachone and menadiol, the k_4 values reflect the E_0' values.

By comparison of the rates of quinol production and oxidation, the redox steady state of the β-lapachone system was calculated. For instance, at 10μM-quinone and a mitochondrial protein concentration of 0.1mg/ml, the rate of quinol production was about 0.18μmol/min per litre (Fig. 5), whereas at 10μM-quinol, the rate of the oxidation was 11.4μmol/min per litre (Table 1), which means that in the steady state, the β-lapachone couple would be 1.6% reduced and 98.4% oxidized. This calculation was confirmed (experimental results not given) by measuring the lack of effect of NADH on the bleach-

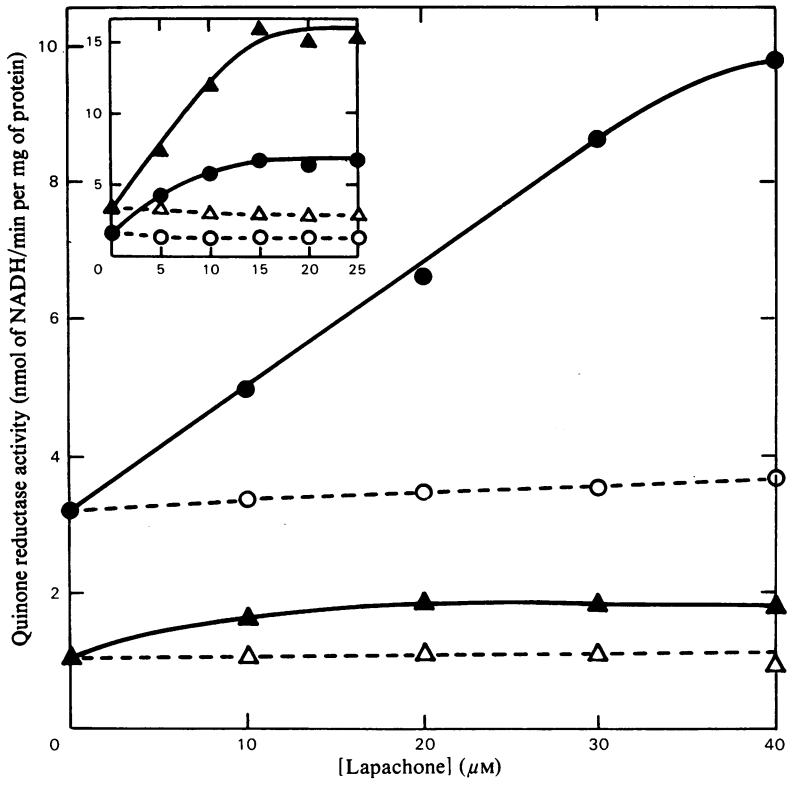

Fig. 5. *Effect of β-lapachone and α-lapachone on the cyanide-insensitive NADH- and NADPH-oxidase activity of the mitochondrial and microsomal fraction of T. cruzi*

The main graph and the inset show the data corresponding to the mitochondrial and microsomal fractions respectively. The incubation mixture contained 50mM-potassium phosphate buffer, pH7.2, and 0.1–0.2mg of protein/ml. The other experimental conditions were as indicated in the Materials and Methods section. ●, NADH and β-lapachone; ○, NADH and α-lapachone; ▲, NADPH and β-lapachone; △, NADPH and α-lapachone.

Table 1. *Second-order reaction constants for O_2^- and H_2O_2 production in the autoxidation reaction of reduced lapachones*

Quinols were prepared as described in the Materials and Methods section, diluted in 50mM-Tris/Mops pH7.4 and used in the concentration range of 1–30μM. The second order reaction of quinols were calculated from the linear relationship between the amount of quinol and the rate of O_2^- and H_2O_2 generation, according to eqns. (1) and (2) (see the text). The oxygen concentration was kept constant. Other experimental details were as indicated previously (Cadenas *et al.*, 1977). E_0' (at pH7.4) calculated from values given by Fieser & Fieser (1960).

	E_0' (mV)	k_3 ($M^{-1} \cdot s^{-1}$)	k_4 ($M^{-1} \cdot s^{-1}$)
Reduced β-lapachone	−17	0.13	5200
Reduced α-lapachone	−120	2.78	23
Menadiol	−60	1.38	5

ing of the 258nm and the 440nm absorption bands of β-lapachone, in the presence of mitochondrial and microsomal preparations.

Effect on growth

Growth of *T. cruzi* epimastigotes was brought practically to a standstill by 5μM-β-lapachone (Fig. 6) since the growth constant k ($k = 0.693/T$; T = generation time) was decreased by a factor of 20. Conversely, α-lapachone was almost inactive, since 30μM-quinone only decreased k by about 20%.

Discussion

T. cruzi epimastigotes are effective H_2O_2 generators. According to our data on specific activity of cellular fractions and distribution of subcellular

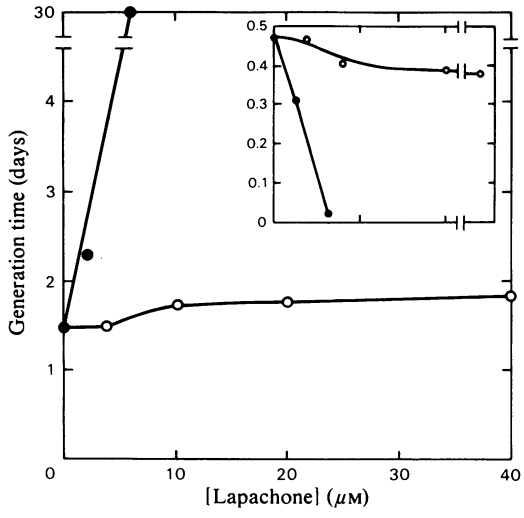

Fig. 6. *Effect of β-lapachone and α-lapachone on growth of T. cruzi epimastigotes*
Experimental conditions were as described in the Materials and Methods section. The inset shows the effect of the quinones on the growth constant, *k*. ●, β-lapachone; ○, α-lapachone.

protein (Boveris & Stoppani, 1977), the epimastigotes would produce, with an adequate substrate supply, about 260nmol of H_2O_2/min per g wet weight, which is about three times as high as the H_2O_2 production by rat liver cells (90nmol/min per g wet tissue; Boveris *et al.*, 1972). The main defence mechanism of *T. cruzi* epimastigotes against the accumulation of the intermediates of the partial reduction of oxygen (O_2^- and H_2O_2) includes two enzymes, namely, superoxide dismutase and peroxidase. Superoxide dismutase is present in the homogenate with an activity equivalent to 0.28μg of bovine superoxide dismutase/mg of protein (Boveris & Stoppani, 1977). The ascorbate-utilizing peroxidase is microperoxisomal, but it is also in the cytosol to a limited extent (Docampo *et al.*, 1976). It must be noted that peroxidase reaction requires one hydrogen donor molecule per H_2O_2 molecule utilized and, consequently, the rate of H_2O_2 detoxication is determined by the rate of hydrogen donor production. *T. cruzi* as other members of the tripanosomatidae family, does not contain catalase (Docampo *et al.*, 1976), and diffusion of H_2O_2 through cell membrane to the extracellular medium as a defence mechanism does not seem to be operative under physiological conditions (Fig. 2). In consequence, catalase-lacking trypanosomes (like *T. cruzi*) should have intracellular H_2O_2 concentrations that far exceed those of mammalian tissues. In this context, Meshnick *et al.* (1977) have reported 70μM-H_2O_2 in *Trypanosoma brucei* (an African trypanosome), approximately 30 times as high as in rat liver, namely 2.6μM (the other report gives H_2O_2 in rat liver as 1–100nM; Oshino *et al.*, 1973). Since H_2O_2 is toxic for *T. cruzi* and other members of the Trypanosomatidae (Fulton & Spooner, 1956), substances that increase H_2O_2 generation or decrease H_2O_2 utilization (Kusel *et al.*, 1973; Boveris & Stoppani, 1977), or that catalyse the homolytic breakdown of H_2O_2 (Meshnick *et al.*, 1977) are potential trypanocidal drugs. No direct demonstration of superoxide toxicity towards *T. cruzi* has been reported. Nevertheless, when ten different β-lapachone-related naphthoquinones were assayed for superoxide-anion generation and toxic action on *T. cruzi*, the correlation coefficient (*r*) was 0.88 ($P < 0.01$), thus suggesting a toxic action of the superoxide anion (Boveris *et al.*, 1978).

Addition of β-lapachone to *T. cruzi* epimastigotes increases the intracellular rate of O_2^- and H_2O_2 generation and releases the oxygen reduction intermediates to the suspending medium. The immediate response indicates a rapid permeation of the quinone through the epimastigote cell membrane. The chemical reactions that would explain the trypanocidal action of β-lapachone (L) could be written:

$$NAD(P)H + H^+ + L \longrightarrow NAD(P)^+ + LH_2 \quad (3)$$

$$LH_2 + L \rightleftharpoons 2LH^\cdot \quad (4)$$

$$LH_2 + O_2 \xrightarrow{k_4} L + H_2O_2 \quad (5)$$

$$LH_2 + O_2 \xrightarrow{k_3} LH^\cdot + O_2^- + H^+ \quad (6)$$

$$LH^\cdot + O_2 \longrightarrow L + O_2^- + H^+ \quad (7)$$

$$O_2^- + O_2^- + 2H^+ \rightleftharpoons H_2O_2 + O_2 \quad (8)$$

$$O_2^- + H_2O_2 \rightleftharpoons O_2 + OH^\cdot + HO^- \quad (9)$$

Reaction (3) is the well known quinone reductase reaction (Crane, 1961; Slater *et al.*, 1961; Brodie, 1965; Ruzicka & Crane, 1971) that takes place in the mitochondrial and endoplasmic-reticulum membranes, where the lipophilic character of β-lapachone molecules should play an important role. Mitochondrial membranes and NADH are more important by far than the endoplasmic reticulum and NADPH as the system that reduces β-lapachone in *T. cruzi*, considering (*a*) the similarity between the effect of β-lapachone on H_2O_2 production by the homogenate and by the mitochondrial fraction, and (*b*) the protein distribution and the specific activity of the respective subcellular fractions. Quinone reduced forms are enzymically oxidized by molecular oxygen, yielding H_2O_2 and O_2^- [reactions (5)–(7)]. With β-lapachone and *T. cruzi* subcellular fractions, quinone reduction [reaction (3)] is slower than quinone oxidation [reactions (5)–(7)], β-lapachone usually being kept in a highly (over 98%) oxidized

state. Semiquinone formation [reaction (4)] is a very rapid process in quinol/quinone mixtures (Michaelis, 1951; Yamazaki & Ohnishi, 1966) and could be even more so when the molecules are bound to a membrane. Incidentally, mitochondrial membranes appear to stabilize ubisemiquinone by binding it to a specific site (Cadenas *et al.*, 1977; Ingledew *et al.*, 1976; Salerno *et al.*, 1977). Subcellular fractions and *T. cruzi* epimastigotes produce and release O_2^- and H_2O_2 in rather similar amounts, implying the function of reactions (6) and (7). Semiquinone autoxidation [reaction (7)] appears to be the main rate-limiting step of this set of reactions as shown by: (*a*) the model autoxidation reaction of mitochondrial ubisemiquinone (Cadenas *et al.*, 1977); (*b*) the fact that O_2^- and H_2O_2 are produced in similar amounts in the mitochondrial samples, although fully reduced β-lapachone mainly generates H_2O_2; (*c*) the detection of the semiquinone free radical in *T. cruzi* epimastigotes treated with β-lapachone (Docampo *et al.*, 1978*a*). Superoxide anion dismutation yields H_2O_2 [reaction (8)]. The Haber–Weiss reaction [reaction (9)] generating either hydroxyl radical (Fong *et al.*, 1973; Zimmerman *et al.*, 1973) or singlet oxygen (Kellog & Fridovich, 1975) seems to be the most reasonable mechanism underlying the complex chain reaction that leads to extensive lipid and organic peroxide formation and to biological damage. In this connection it seems pertinent to recall that *T. cruzi* epimastigotes supplemented with β-lapachone show increased lipid peroxidation (Docampo *et al.*, 1978*a*). The metabolic and ultrastructural alterations observed in β-lapachone-treated *T. cruzi* cells (Docampo *et al.*, 1977; Cruz *et al.*, 1978) could be easily accounted for by extensive organic and lipid peroxide formation, leading to enzyme inactivation and membrane damage.

Another amphipathic naphthoquinone, vitamin K_1, has been reported to act as electron carrier in artificial bulk membranes (Anderson *et al.*, 1976), an experimental fact that supports the hypothesis that quinones may interfere in proton conductance in the mitochondrial membranes and, consequently, uncouple oxidative phosphorylation, especially in the ubiquinone–cytochrome *b* region (Mitchell, 1975*a*,*b*). β-Lapachone does indeed uncouple oxidative phosphorylation in rat liver mitochondria; however, the effective uncoupling concentration (20 μM; Docampo *et al.*, 1977) is about five times as high as that (4 μM) which produces maximal stimulation of H_2O_2 release (Fig. 3) and completely inhibits *T. cruzi* growth (Fig. 6). Ultrastructural alterations in β-lapachone-treated *T. cruzi* cells were also different to those observed in epimastigotes treated with other uncoupling agents such as 2,4-dinitrophenol (Docampo, 1977). So, the uncoupling activity of β-lapachone can be excluded as an explanation of the present results.

It is noteworthy, that other bactericidal and cytotoxic drugs, such as streptonigrin (Gregory & Fridovich, 1973), adriamycin (Thayer, 1977), toxoflavin (Latuasan & Berends, 1961), mitomycin C (Tomasz, 1976) etc., probably share with β-lapachone the same general mechanism of biological action. Specifically, with respect to trypanosomatidae, naphthoquinones, by increasing the rate of H_2O_2 generation and haematoporphyryn (Meshnick *et al.*, 1977) by increasing hydroxyl radical formation, provide molecular models that could develop into practical trypanocidal drugs.

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